Making a mark

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Making a mark: the role of RNA modifications in plant biology

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Plants coordinate their growth and development through complex regulatory networks involving changes in the expression of thousands of genes. Many developmental pathways are regulated at the level of messenger RNA (mRNA) through alternative choices in mRNA processing. These choices can have consequences for the localization, stability or translatability of mRNAs. One of the key ways in which RNAs are processed is by the methylation of the RNA base adenosine – a modification known as m^6A. Even though it was first discovered in the 1970s, the biological significance of m^6A marks has only recently become clear. In this feature article, we identify the factors controlling the writing and reading of m^6A modifications in plants. We also highlight some of the features of plant development that depend on m^6A and explore the recently discovered molecular mechanisms that use m^6A to control development or response to environmental stress.

The importance of RNA processing and modification

Messenger RNA (mRNA) is often presented as a copy of the information contained within a gene – an intermediate step in the path to making proteins from DNA. However, during transcription, premature mRNA (pre-mRNA) molecules undergo a range of processing events that shape mature mRNAs. These include splicing of exons and introns, selection of 5′ start and 3′ end sites and the addition of a poly(A) tail that can vary in length. The choices made during pre-mRNA processing can influence the fate of mRNAs. Alternative splicing can change the sequence of the protein that each mRNA codes for, increasing the diversity of proteins that can be produced from any one gene. By adding or removing regulatory elements to which proteins can be bound, the fate of mRNAs can be altered, e.g., they can be stabilized, or targeted for degradation. In plants, RNA processing is crucial in the regulation of key developmental pathways. For example, plants must integrate multiple environmental cues including day length and temperature to choose when to produce flowers. Many of the proteins that tune this carefully balanced process do so by altering the processing of mRNAs encoding core transcription factors.

RNA molecules can also be processed by the covalent addition of chemical modifications to RNA bases. The most common internal modification to mRNAs is the methylation of adenosines at the N^6 position, often referred to as m^6A. The process by which RNA is methylated appears to be conserved in plants and animals. However, emerging evidence points to key differences between the two kingdoms in the way m^6A affects RNA metabolism.

How are plant mRNAs modified?

Methyl groups are added to adenosine ribonucleotides by a group of catalytic and regulatory proteins referred to as the m^6A writer complex (Figure 1). The key proteins in this complex are methyltransferases: MTA, the main catalytic component; and MTB, a related protein whose catalytic activity is unconfirmed. Additional proteins co-purifying with MTA and MTB, named VIRILIZER, FIP37 and HAKAI, are required for normal levels of m^6A. The exact functions of these regulatory proteins are not yet known. The writer complex appears to recognize, bind and methylate mRNAs whilst transcription is still underway. Recent technological advances, which identified m^6A marks dependent on the writer complex component VIRILIZER, showed that in *Arabidopsis* methylated adenosines are almost exclusively located within 3′ untranslated regions. These regions do not contribute to the protein-coding potential of mRNAs and are instead thought to have regulatory functions in mRNA metabolism.

Once added to 3′ untranslated regions, m^6A might alter the fate of mRNAs by changing the physical properties or structure of the RNA. Alternatively, it could act as a platform for the binding of regulatory proteins known as m^6A readers (Figure 2). The majority of known m^6A readers contain a specialized YTH domain which is able to bind to m^6A directly (Box 1). In *Arabidopsis*, 13 proteins with YTH domains have been identified. The best characterized of these, ECT2, ECT3 and ECT4, are localized in the cytoplasm and have long disordered tails which might act as scaffolds to recruit other proteins to methylated mRNAs. Plants differ from animals in that a conserved protein in the regulation of cleavage and

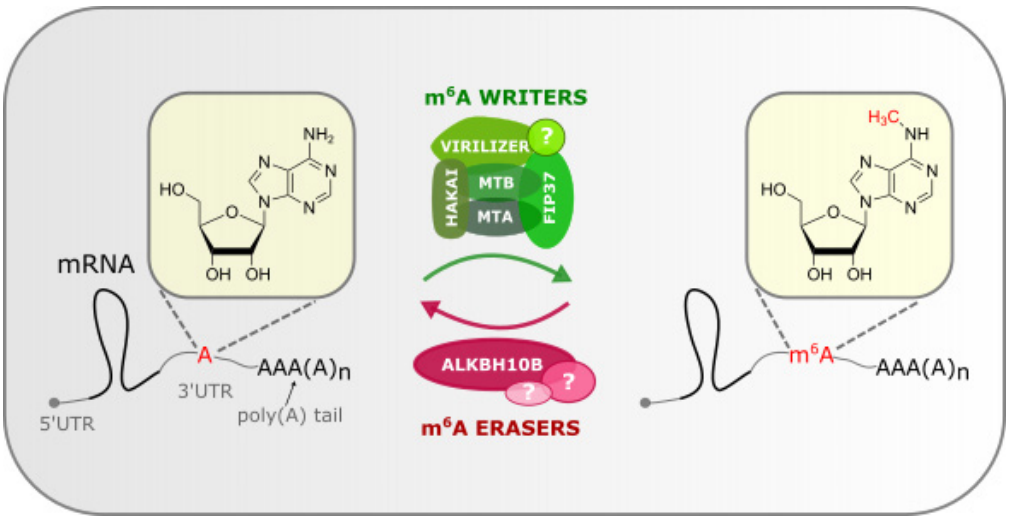

Figure 1. Writer complex is involved in methylation of adenosines within mRNAs. m^{6}A is formed by adding methyl groups (–CH_3) at the N6 position of adenosines located in the 3′ untranslated regions (3′ UTRs) of mRNAs. Methylation is catalysed by the writer complex (green) and might be removed by eraser proteins (red). Adapted from Arribas-Hernández et al. (2020) https://doi.org/10.1104/pp.19.01156

polyadenylation, CPSF30, has gained a YTH domain (Figure 3). CPSF30 is important for recognizing the motifs that signal the end of an mRNA and recruiting the machinery that cuts it and appends a poly(A) tail. This suggests that m^{6}A, via interaction with CPSF30, could play a role in regulating the formation of mRNA ends in plants.

The discovery of a class of proteins which are able to remove m^{6}A from mRNAs (referred to as erasers) has led to the idea that m^{6}A might be added to or removed from mRNAs in response to different cellular and environmental stimuli. However, to date, only two mRNA m^{6}A demethylases, ALKBH9B and ALKBH10B, have been described in *Arabidopsis*.

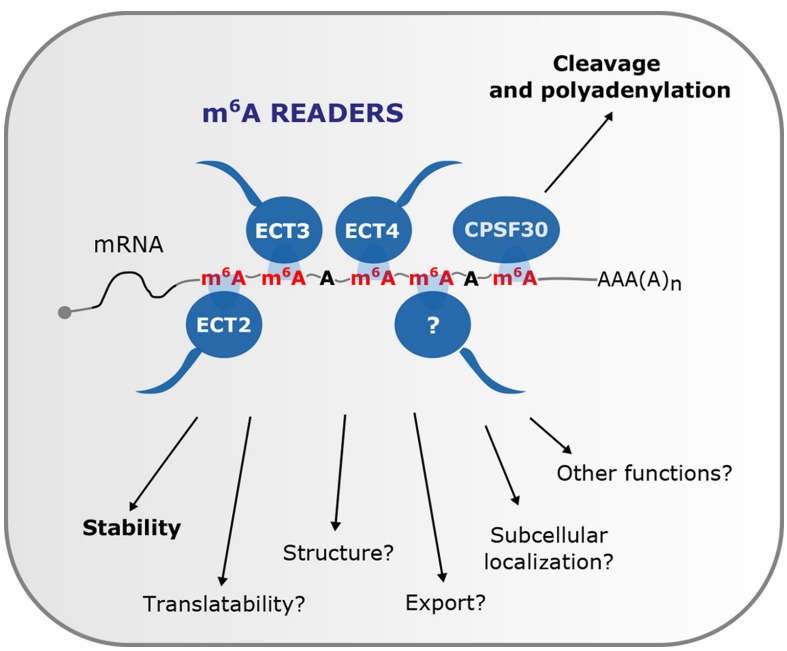

Figure 2. Possible functions for m^{6}A in mRNA metabolism. Experimentally verified (bold) and potential functions for m^{6}A in mRNA metabolism. These functions may depend on the m^{6}A recognition and binding by reader proteins. Adapted from Arribas-Hernández et al. (2020) https://doi.org/10.1104/pp.19.01156

Box 1. YTH domains

- First identified in 2002 in the mammalian splicing factor YT521-B.
- Examples are found across the eukaryotic domain.
- Interact with mRNAs using conserved aromatic residues which form an m^6A-binding pocket (Figure 3).
- Plant genomes tend to contain large and variable numbers of YTH domain proteins, some of which may be functionally redundant.

What is the biological relevance of m^6A modification?

The importance of m^6A is highlighted by the fact that *Arabidopsis* mutants defective in the writer MTA exhibit abortion of embryos early in development. Only mutant alleles that retain at least a low level of m^6A can develop further. Most viable m^6A writer mutants, e.g. *mta, virilizer* and *fip37*, are therefore weak alleles with 5%–15% of the m^6A detected in normal plants. These residual m^6A levels allow plants to grow, albeit with severe defects, enabling the study of the effects of low methylation. Interestingly, complete loss of function of HAKAI is not embryonic lethal. In these mutants, the m^6A level is reduced by only 35% compared to normal plants, with no significant plant growth defects detected.

m^6A is not only required for the growing embryo, but is also important for later stages of plant development. Plants with mutations in genes encoding m^6A writers have stunted growth and defective organ definition. Issues with cell differentiation lead to a bushier appearance and abnormal formation of leaves. Root growth, architecture and vascular system development are also affected. Loss of m^6A also seems to cause problems with measuring day length: in *virilizer* mutants, the circadian period is prolonged from 24 to 25 hours. Mutations in individual m^6A reader proteins generally lead to less severe defects than mutations disrupting m^6A writing. This suggests that reader proteins share some redundant functions. When multiple readers are mutated at the same time, however, problems start to emerge. Double mutants of the m^6A reader proteins ECT2 and ECT3 exhibit defects in the morphogenesis of leaf trichomes (specialized structures on leaf surfaces which are important for reducing water loss and providing defence against insects), as well as delayed and aberrant leaf formation. These deformities are even more visible in triple mutants lacking ECT2, ECT3 and ECT4, and begin to resemble the appearance of a weak m^6A writer mutant *mta*. This suggests that the defects displayed in m^6A writer mutants may be caused by a loss of reader binding sites.

How does m^6A affect the fate of plant mRNAs?

The huge impact that loss of m^6A has on plant development raises questions regarding the molecular mechanisms which utilize m^6A. High throughput sequencing of

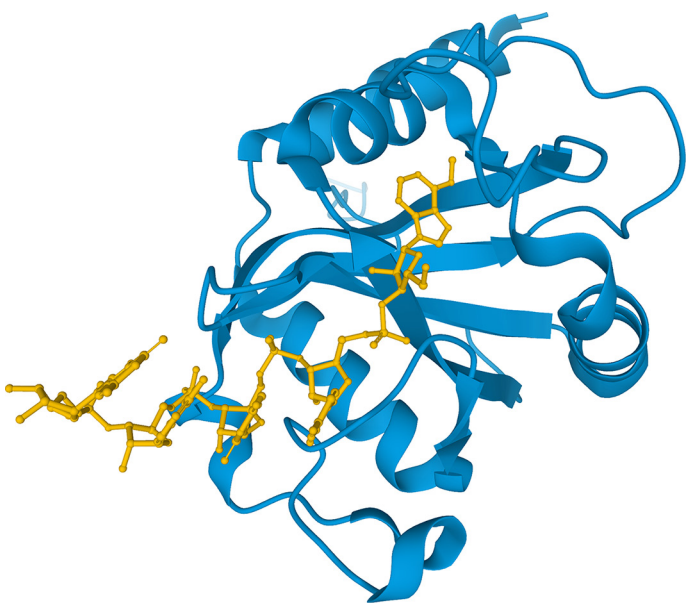

Figure 3. The crystal structure of the *Arabidopsis* CPSF30 YTH domain (blue), in complex with an m6A-modified RNA (yellow). Image created using Mol* using structural data deposited by Wu, B.X., Nie, H.B., Li, S.S., Patel, D.J. (2019). RCSB PDB, ID: 5ZUU. DOI: 10.2210/pdb5ZUU/pdb

the global mRNA pools extracted from *mta*, *fip37* and *virilizer* mutants has identified a reduction in the relative abundance of mRNAs which would be methylated under normal conditions. This indicates that in plants, m^6A might control the gene expression by stabilizing mRNAs, in contrast to mammals, where it appears to speed up their degradation. Similar observations have been made for some m^6A readers – complete inactivation of ECT2 also causes destabilization of methylated mRNAs. This finding demonstrates that the stabilizing effect of m^6A can be explained by the binding of m^6A by reader proteins.

Only slight changes in mRNA splicing have been observed in mutants of the writer complex. However, in *virilizer* and *fip37* mutants, problems with the proper formation of mRNA 3′ ends have been detected. In these mutants, loss of m^6A causes a global switch to mRNAs with shorter 3′ untranslated regions. The mechanism for this is still unclear, but one hypothesis is that it involves the recognition of m^6A by the RNA processing factor CPSF30. Why plants would want to control mRNA length in this way is also not yet known. However, untranslated regions often contain regulatory sequences which might be included or excluded by changes in length. This may impact the repertoire of regulatory proteins binding to mRNAs, potentially affecting their stability, localization, translatability or folding.

m^6A in a changing environment

Plants adapt to changes in their environment by reprogramming gene expression. m^6A is a potential candidate for the control of stress response, since recognition of m^6A marks by readers could target specific mRNAs for rapid post-transcriptional control. However, there is currently only a small amount of work to support this hypothesis. It has been shown that the expression of genes encoding m^6A writers and readers is affected by abiotic (e.g., heat and salinity) and biotic (pathogen-triggered) stresses. For example, in tobacco plants which have been infected by the tomato mosaic virus, the abundance of mRNAs coding for m^6A writers decreases, whilst the abundance of potential erasers increases. There are also indications that m^6A within mRNAs encoding salt stress response proteins increases the levels of these mRNAs upon salinity stress treatment.

In general, m^6A appears to be a feature of mRNAs which are highly abundant and efficiently translated into proteins. These proteins are involved in translation and key growth and development processes such as photosynthesis and respiration. In stress conditions, a large proportion of such mRNAs are rapidly targeted for degradation or translational silencing as cells halt their normal growth. During heat stress, *Arabidopsis* ECT2 relocalizes from the cytosol to cytoplasmic foci called 'stress granules'. These are dense aggregations composed of proteins and RNA molecules, which are prevented from being translated into new proteins. Similar relocalization events have also been identified for ECT2, ECT4 and ECT3 under osmotic stress. This suggests that the selective deposition of m^6A could have a role in controlling which mRNAs are relocalized during stress. However, the lack of experiments to determine how mutants lacking m^6A function in stress conditions means that there is not yet an explanation as to how this affects plant stress adaptation.

The missing pieces of the puzzle

Despite the work that has been done to identify m^6A writers, readers and erasers, it is far from clear if all the factors which play a role in m^6A biology have been identified. We understand little of how the regulatory proteins which are associated with the writer complex function, nor whether there are other RNA methyltransferases targeting different RNA species. Characterization of m^6A readers lags further behind. Most have only been identified by the presence of a YTH domain, and so their ability to bind m^6A needs to be experimentally verified. It is also possible that other domains can bind m^6A, and that the repertoire of m^6A readers is broader than currently thought. The least explored class of m^6A regulating proteins are the erasers. It is not clear how many m^6A erasers exist in plants, whether they are able to efficiently remove m^6A from mRNAs, or what their biological importance is. Getting answers to these questions would help to resolve the controversial question of whether m^6A is a dynamic mark.

In addition to m^6A, more than 150 different modifications have been identified in eukaryotic and prokaryotic RNAs. It is unclear which of these occur within plant mRNAs – studies have so far only described 5-methylcytosine (m^5C) in *Arabidopsis* and rice. Technologies such as nanopore direct RNA sequencing, which has been recently used to identify *Arabidopsis* m^6A, could in future help us to recognize all plant mRNA modifications and map their positions in the transcriptome.

From the current evidence, it is clear that m^6A modifications are important for regulating the processing and stability of plant mRNAs. In future, a clearer understanding of the mechanisms behind this regulation might help us to improve the design and productivity of plant transgenes, with applications in pharmaceutical or vaccine production. m^6A is also an interesting candidate for the regulation of plant stress responses, and there may be further applications in adapting plants to environmental change. This overlooked layer of gene regulation promises to yield new insight into the life of plants. ■

Further reading

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